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Synergic bactericidal activity of novel antibiotic combinations against extreme drug resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*

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The emergence of extreme drug resistant (XDR) *Pseudomonas aeruginosa* and *Acinetobacter baumannii* represent a major problem in health care settings. Colistin is the only therapeutic option available for treatment of infections caused by such pathogens. Worryingly, colistin resistance recently emerged which may lead to the return to the pre antibiotic era. The aim of the present study was to assess for the first time the bactericidal activity of four novel triple antibiotic combinations, including imipenem (IMP), amikacin (AK), cefepime (CEF) and tigecycline (TIG) against four XDR *P. aeruginosa* and *A. baumannii* clinical pathogens. Antibiotic combinations were evaluated by time-kill assay at the breakpoints. Three combinations (IMP/TIG/AK, TIG/AK/CEF and IMP/TIG/CEF) showed significant bactericidal activity against XDR *A. baumannii* isolates. Only two combinations (IMP/AK/CEF and IMP/TIG/AK) displayed remarkable killing against XDR *P. aeruginosa* isolates. These finding revealed that these triple antibiotic combinations are attractive therapeutic options for treatment of infections caused by XDR pathogens and could be utilized as an alternative to colistin. Further studies are warranted to assess the clinical outcomes of such combinations.

Key words: Extreme drug resistant (XDR), *Pseudomonas aeruginosa, Acinetobacter baumannii*, antibiotic combinations, bactericidal activity.

INTRODUCTION

Antimicrobial resistance represents a global health threat and has reached crisis points in different areas around the world (Lee et al., 2011). Recently, many extreme drug resistant (XDR) pathogens were isolated. These pathogens showed resistance to most available antimicrobial agents. Infections with such pathogens are associated with high rate of morbidity and mortality (Papp-Wallace et al., 2011). In the past decade, many XDR *A. baumannii* (XDR-AB) and *P. aeruginosa* (XDR-PA) were isolated locally and globally (Johnson and Woodford, 2013; Alsultan et al., 2013).

A bacterial isolate of *A. baumannii* or *P. aeruginosa* is considered as XDR when it remains susceptible to only one or two antimicrobial categories (aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, penicillins plus β-lactamase inhibitors, polymyxins, etc) (Magiorakos et al., 2012). In addition to its intrinsic resistance, *P. aeruginosa* has acquired resistance through production of β-lactamases and carbapenemases, overexpression of efflux pumps and

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reduction in porin channels while *A. baumannii* have developed resistance to several antimicrobial agents via production of aminoglycoside-modifying enzymes, ESBLs, and carbapenemases, in addition to target-site alteration such as changes in outer membrane proteins, penicillin binding proteins and topoisomerases (Slama, 2008).

Due to the dearth of antimicrobial effect with novel mechanisms of action, very few options remain for the treatment of infections caused by such XDR pathogens (Isturiz, 2008). Colistin became the last-line therapy for treatment of serious infections caused by such XDR pathogens (Lim et al., 2010; Breilh et al., 2013). Unfortunately, resistance to colistin has been reported all over the world (Marchaim et al., 2011; Mammina et al., 2012; Lesho et al., 2013) which should be a reason for rationalization of use of colistin to reduce the rate of emergence of such resistant superbugs and panresistant pathogens which may return the world to the pre antibiotic era (Gould, 2008; Rai et al., 2013). Therefore, prolonging the effectiveness of currently available antimicrobial agents through antibiotic combinations is the only available option (Lee et al., 2011).

Antibiotic combinations therapy may provide a successful strategy for treatment of patients infected with XDR pathogens. These antibiotic combinations may be the only available option until discovery of new active antimicrobial agent. The potential increased value of double and triple antibiotic combination therapy over monotherapy for treatment of infection caused by such pathogens was extensively studied (Rahal, 2006; Lim et al., 2011; Albur et al., 2012; Urban et al., 2010). Most of these antibiotic combinations are colistin based (Cai et al., 2012). As colistin is the last therapeutic option for treatment of infections with XDR pathogens and due to its high toxicity and the rapid emergence of colistin resistance as mentioned above, it is recommended to look for novel antibiotic combinations which avoid the use of colistin and in the same time able to fight such pathogens.

The aim of the present study was to assess the *in vitro* bactericidal activity of four novel three-antibiotic combinations including TIG, IMP, AK and CEF against XDR-AB and XDR-PA by time-kill assay.

MATERIALS AND METHODS

Bacterial isolates

Four non repetitive XDR clinical isolates (two *P. aeruginosa* and two *A. baumannii*) were identified using BioMerieux VITEK 2 compact automated system (BioMerieux, Marcy l'Etoile, France) according to the instructions of the manufacturer. The tested *P. aeruginosa* and *A. baumannii* clinical isolates were collected from sputum and blood specimens, respectively.

Detection of Metallo-β-lactamase (MBL) production

Imipenem/imipenem plus EDTA (IP/IPI) Etest strips (AB Biodisk, Solna, Sweden) were used to test the ability of the XDR clinical isolates to produce MBL as described by the manufacturer. The production of MBL is confirmed when the ratio of the minimum inhibitory concentration of IMP to IMP-EDTA is ≥ 8 or the development of phantom zone.

Susceptibility testing

The resistance pattern of the tested isolates was determined by VITEK 2 compact automated system against twenty different antimicrobial agents using AST-N116 cards in accordance with the guidelines of the manufacturer. The tested antimicrobial agents were: ampicillin, ampicillin/sulbactam, piperacillin, piperacillin/-tazobactam, cefazolin, cefuroxime, cefuroxime axetil, cefoxitin, cefpodoxime, cefotaxime, ceftazidime, cefepime, imipenem, meropenem, gentamicin, tobramycin, ciprofloxacin, levofloxacin, tigecycline and sulphamethoxazole/trimethoprim. Disc diffusion assay was used to test the resistance of the isolates to amikacin (AK) and to confirm the resistance to imipenem (IMP), cefepime (CEF) and tigecycline (TIG). Antibiotic discs were purchased from Oxoid Ltd, UK. The minimum inhibitory concentrations (MICs) of the four tested antibiotics in addition to colistin were determined by Etest strips (AB Biodisk, Solna, Sweden) as the manufacturer guidelines. All experiments were carried out in duplicate.

Time-kill assay

Time-kill curve analysis was used to evaluate the bactericidal activities of four different triple antibiotic combinations against the four tested XDR isolates (Pillai et al., 2005). The four tested triple antibiotic combinations are: (i) IMP/TIG/AK; (ii) TIG/AK/CEF; (iii) IMP/TIG/CEF and (iv) IMP/AK/CEF. All experiments were carried out in duplicate using Mueller-Hinton broth (MHB, Oxoid Ltd, UK). Each antimicrobial agent was used at its breakpoint concentration (2, 4, 8 and 16 mg/L for TIG, IMP, CEF and AK, respectively). The initial inoculum was adjusted at about 10^6 CFU/ml. Suitable growth controls lacking the antibiotics were included in the experiment. The experiments were carried out as recently described (Aboulmagd et al., 2013).

Tested isolates were exposed to the different combinations at the above mentioned concentrations and samples were withdrawn at 0 h and after 3, 6 and 24 h contact time. After suitable dilutions, aliquots were plated onto Mueller Hinton agar and colonies were counted after 24 h incubation at 37°C. The time-kill kinetics was demonstrated by plotting the log number of survivors per ml (log CFU/ml) against the time. The combination was considered bactericidal when ≥ 3 $\log_{10}$ reduction in CFU/ml (≥ 99.99 % killing) from the initial count was recorded in 24 h while synergy was defined as a ≥ 2 $\log_{10}$ decrease in CFU/ml (≥ 90 % killing) by the drug combination when compared with the most active drug after exposure for 24 h.

The lower limit of detection for the bacterial colony counts was 10^2 CFU/ml. Any colony count lower than this limit was rounded to 10^2 CFU/ml.

RESULTS

VITEK 2 compact automated system was used for identification of four non-duplicate XDR *A. baumannii and P. aeruginosa* pathogens (two isolates each) and for determination of their resistance pattern against twenty different antimicrobial agents. The resistance of the isolates to AK was determined by disc diffusion method and MIC determination of the four antibiotics was

Table 1. Minimum inhibitory concentration (MIC) values of imipenem, amikacin, cefepime, tigecycline and colistin against four tested XDR isolates.

Clinical isolate†	MIC (mg/L)				
	IMP‡	AK	CEF	TIG	COL
XDR-PA 98	24	128	> 256	16	0.25
XDR-PA 12	16	48	> 256	32	0.75
XDR-AB 23	24	32	> 256	32	0.5
XDR-AB 17	> 32	64	> 256	64	0.75

†The tested isolates showed resistance to all tested antimicrobial agents mentioned in the "Material and Methods" ‡Abbreviations: IMP, imipenem; AK, amikacin; CEF, cefepime; TIG, tigecycline; COL, colistin.

Table 2. Logarithmic changes (Log_{10} CFU/ml) from the initial inoculum of time-kill assay after 24 h exposure to four different triple antibiotic combinations**.**

Isolate code	Mean Log_{10} CFU/ml changes† after 24 contact time			
	TIG/AK/CEF‡	IMP/AK/CEF	IMP/TIG/AK	IMP/TIG/CEF
XDR-PA 98	+ 3.1	- 2.6	- 2.1	+ 2.3
XDR-PA 12	+ 2.6	- 3.2	- 2.3	+ 2.1
XDR-AB 23	- 2.1	+ 3.1	- 2.4	- 3.2
XDR-AB 17	- 3.9	+ 2.5	- 4.1	- 2.6

†Positive values represent an increase above the initial inoculum while negative values represent a reduction. ‡Abbreviations and used concentrations: TIG: tigecycline (2 mg/L); AK: amikacin (16 mg/L); CEF: cefepime (8 mg/L); IMP: imipenem (4 mg/L).

performed by Etest. The isolates showed resistance to all tested antibiotics (mentioned in the Material and Methods) including those under investigation in this study (IMP, AK, CEF and TIG). All tested isolated were susceptible to colistin.

Table 1 shows the minimum inhibitory concentration (MIC) values of the four tested antimicrobial agents in addition to colistin against XDR isolates. The MIC values of IMP, AK and TIG ranged 16 - >32 mg/L, 32 - 128 mg/L and 16 - 64 mg/L, respectively, while the MIC values of CEF and colistin were >256 and ≤ 0.75, respectively, against all isolates.

Time-kill assay was used to evaluate the bactericidal activities of the three-antibiotic combinations under investigation and the logarithmic changes (Log_{10} CFU/ml) from the initial inoculum were shown in Table 2 and Figures 1 and 2. IMP/TIG/AK combination was the only triple therapy which showed significant killing rate against both tested species after 24 h exposure where bactericidal activity (4.1 Log_{10} reduction) was shown against XDR-AB 17 (Figure 1A) and synergy (> 90% killing) was recorded against the rest of the isolates (Figures 1B and 2).

After 3 h exposure, rapid bactericidal activity was demonstrated by IMP/TIG/AK and TIG/AK/CEF combinations against XDR-AB 17 (Figure 1A). This bactericidal effect was maintained for 24 h (Table 2). On the other hand, IMP/TIG/CEF, IMP/TIG/AK and TIG/AK/CEF combinations showed 3.2, 2.4 and 2.1 log_{10} reductions, respectively, by 24 h against XDR-AB 23 (Table 2, Figure 2B).

As shown in Table 2, IMP/AK/CEF and IMP/TIG/AK showed remarkable killing against both XDR-AP isolates (2.1-3.2 Log_{10} reduction). On the other hand, TIG/AK/CEF and IMP/TIG/CEF combinations achieved 99.99% killing against XDR-PA 12 by 3 h contact time (Figure 2B) but regrowth was recorded after 24 h.

DISCUSSION

The emergence and worldwide distribution of XDR pathogens represent a serious problem facing the health care professionals (Paterson and Doi, 2007). Moreover, multi drug resistant *P. aeruginosa* and *A. baumannii* have been recognized in health care settings as a cause of serious infections associated with high mortality rate. These infections are very difficult to treat because very limited effective therapeutic options are available for combating XDR pathogens (Bassetti et al., 2011). This situation represents a dramatic challenge to clinicians and has been forced them to turn to colistin (Lim et al.,

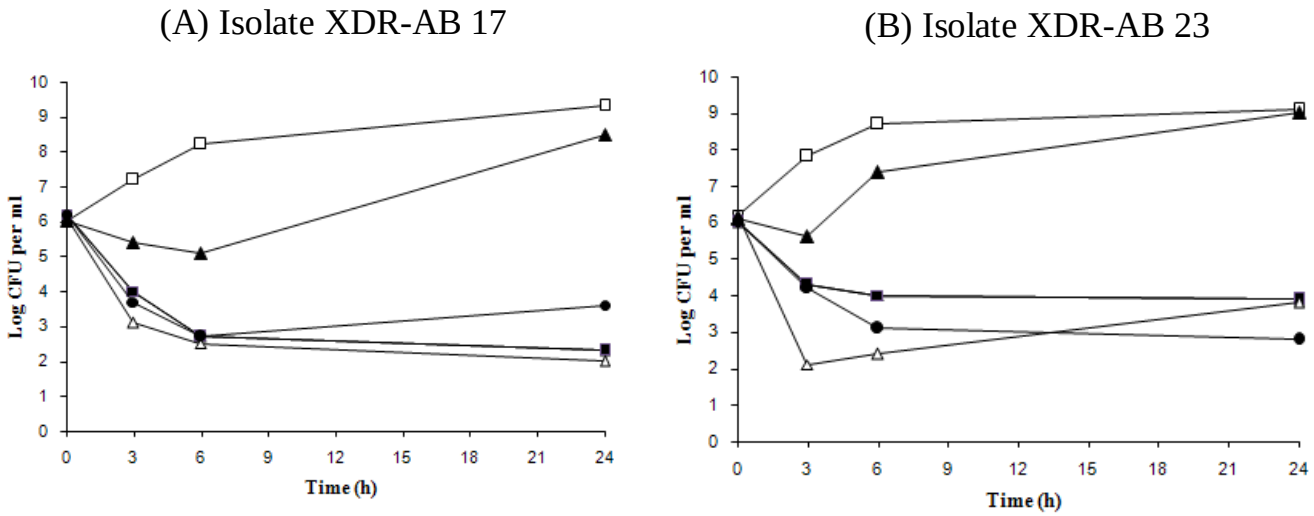

Figure 1. Time-kill kinetics of four different triple antibiotic combinations against (A) XDR-AB 17 and (B) XDR-AB 23. □, Growth control; ■, TIG/AK/CEF; ▲, IMP/AK/CEF; △, IMP/TIG/AK; ●, IMP/TIG/CEF. The concentrations of the antimicrobial agents and the abbreviations were mentioned in the Material and Methods.

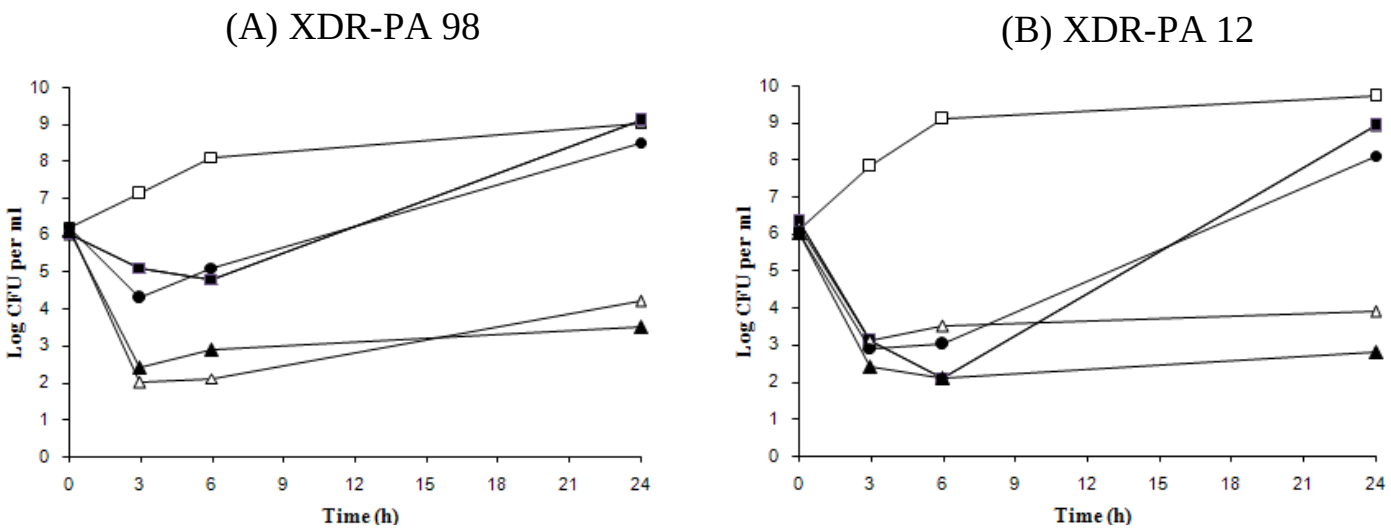

Figure 2. Time-kill kinetics of four different triple antibiotic combinations against (A) XDR-PA 98 and (B) XDR-PA 12. □, Growth control; ■, TIG/AK/CEF; ▲, IMP/AK/CEF; △, IMP/TIG/AK; ●, IMP/TIG/CEF. The concentrations of the antimicrobial agents and the abbreviations were mentioned in the Material and Methods.

2010; Cai et al., 2012). Unfortunately, resistance to colistin was recorded worldwide (Marchaim et al., 2011; Mammina et al., 2012; Lesho et al., 2013) which may indicate a return to the pre antibiotic era (Paterson and Lipman, 2007). Therefore, rationale use of colistin is highly recommended whenever possible to reduce the emergence of resistance to such antibiotic and the development of novel antibiotic combinations is essential and studies in this field should be taken seriously (Papp-Wallace et al., 2011).

The aim of the current study was to assess the bactericidal activity of four novel triple antibiotic combinations against four XDR *A. baumannii* and *P. aeruginosa* isolates. The four triple antibiotic combinations were: IMP/TIG/AK, TIG/AK/CEF, IMP/TIG/CEF and IMP/AK/CEF. Each antimicrobial agent was used at its antibiotic breakpoint. To the best knowledge of the authors, the efficacy of these four triple combinations was not assessed before.

As the four tested isolates in this study were resistance to all antimicrobial agents, single agents did not exhibit any bactericidal activity against tested stains at the anti-

biotic breakpoints. Moreover, the results of bactericidal activity of two antibiotic combinations of the four tested antibiotics at such concentrations against XDR tested isolates were insignificant (data not shown). Therefore, three-antibiotic combinations were assessed aiming to achieve efficient bactericidal activity (high and rapid killing rate).

Our data revealed that IMP-based triple combinations with AK/CEF or TIG/AK achieved > 90% killing after 24 h exposure against both XDR-PA isolates. On contrary, TIG-based combinations with AK/CEF and IMP/CEF were totally ineffective against such isolates. In addition, three of the tested triple therapy showed remarkable reduction in the colony count against XDR-BA tested ranged from 4.1 $\log_{10}$ reduction (IMP/TIG/AK against XDR-BA 17) to 2.1 $\log_{10}$ reduction (TIG/AK/CEF against XDR-AB 23). Interestingly, the bactericidal activity demonstrated by the different triple antibiotic combinations against the XDR pathogens was achieved at the antibiotic breakpoints despite the resistance to each of the three antibiotics singly. These findings need further research to understand the mechanism by which such antibiotics acted synergistically to achieve significant bactericidal activity against completely resistant pathogens.

Combination of IMP/TIG/AK was the only combination which showed significant synergism and bactericidal activity against all tested pathogens. The effect of this combination sustained 24 h against XDR-PA and longer against XDR-AB (data not shown). Therefore, this combination may be an important treatment modality and could be used empirically for combating infections cause by XDR pathogens.

The resistance pattern of the tested isolates revealed that *A. baumannii* and *P. aeruginosa* pathogens displayed resistant to the twenty tested antimicrobial agents in addition to AK (data not shown). Worryingly, XDR in Gram negative bacteria is being reported with increasing rate recently in our laboratory (Alsultan et al., 2013), locally (Al-Agamy et al., 2011; Memish et al., 2012) and globally (Paterson and Doi, 2007; Livermore, 2009). Therefore, efforts to ensure appropriate antibiotic use in hospitals are critical to slow the emergence of XDR which should be matter of concern to clinicians and health authorities. In addition, strict infection control measures and antibiotic stewardship programs should be implemented to minimize the emergence and spread of antibiotic resistance (Lee et al., 2013).

Conclusion

The triple antibiotic combinations assessed in the present study for the first time could be attractive options and salvage therapies for treatment of XDR pathogens where significant bactericidal activity was achieved at the breakpoints and within the clinically achievable serum levels. Further studies are warranted to evaluate the clinical outcomes of these triple therapies. Moreover, these combinations represent good novel effective therapeutic options which could be used as an alternative for colistin to treat infections caused by such pathogens, reduce the emergence of colistin resistance and avoid the serious side effects of colistin such as nephrotoxicity.

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